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Degradation of Maternal Cdc25c During the Maternal to Zygotic Transition Is Dependent Upon Embryonic Transcription

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ABSTRACT To gain a better understanding of the molecular differences that may contribute to cleavage arrest and the poorer development associated with laboratory produced embryos, a series of experiments were conducted to quantitate the message levels of the cell cycle controller cdc25c, over the maternal to zygotic transition (MZT) in 4-cell in vivo- and in vitro-derived porcine embryos. The experiments were designed to measure both maternal and embryonic derived cdc25c transcripts by quantitative reverse transcription-competitive polymerase chain reaction (RT-cPCR), determine the point of the transition to zygotic genome activation, and study the interaction between initial embryonic transcription and maternal cdc25c degradation. Analysis of in vivo- and in vitro-derived embryos revealed no difference in cdc25c message level for any of the times P4CC ($P > 0.05$). Comparison of control embryos from 5- to 33-hr P4CC revealed a reduction in transcript quantities in the 10-hr P4CC group that was maintained at later time points ($P < 0.05$). Embryos cultured in the RNA polymerase inhibitor, α-amanitin, from cleavage to 5-, 10-, 18-, 25-, or 33-hr P4CC displayed no difference in cdc25c levels when compared to controls at similar time points ($P > 0.05$). However, if embryos were first exposed to α-amanitin after 18-hr P4CC with this treatment continuing to 33 hr, the levels of cdc25c transcript were reduced ($P < 0.04$) when compared to those embryos that were first exposed to the inhibitor at either 5- or 10-hr P4CC. This finding and the comparison of these same embryos to the 0–33-hr α-amanitin and control groups allowed us to conclude that cdc25c transcription began between 10- and 18-hr P4CC, with the degradation of maternal cdc25c message dependent on transcriptional initiation.
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INTRODUCTION

The initial activation of the embryonic genome varies between species with the period encompassing this event called the maternal to zygotic transition (MZT). This transition is characterized by changes in both the origin and quantity of mRNA present in the developing embryo. Specifically, the MZT is the period when control of embryonic development is transferred from stored to newly synthesized, embryonic-derived transcripts.

Interestingly, the time of zygotic genome activation (ZGA), which occurs during the MZT, coincides with what is known as the "in vitro block" and is manifested as a developmental arrest during culture under sub-optimal conditions. This correlation between the "in vitro block" and the MZT, as well as the overall reduced quality of in vitro-derived embryos when compared to their in vivo contemporaries, have led us to explore the relationship that exists between these embryos and the quantities of key cell cycle transcripts thought to play a major role during this transitional period.

The MZT is characterized by a large loss of maternally derived transcripts that were originally synthesized during a 2–3-week growth phase in the developing oocyte (Moore and Lintern-Moore, 1978; Wassarman and Kinloch, 1992). This loss approaches approximately two-thirds of all mRNA species by the 2-cell stage in murine embryos (Bachvarova and DeLeon, 1980; Clegg and Piko, 1983) when the MZT occurs (Schultz, 1993). Furthermore, this large mRNA loss is followed by the incorporation of [^{3}H]uridine into newly synthesized transcripts from the activated embryonic genome (Clegg and Piko, 1983). Therefore, both maternal mRNA degradation and the original synthesis of embryonic transcripts affect the timing of the MZT.

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Unlike the mouse, major activation of the embryonic genome at the MZT occurs later in development for the domestic food animals. The porcine MZT occurs during the 4-cell stage (Kopecny, 1989; Prather, 1993), while the cow (Frei et al., 1989) and sheep (Crosby et al., 1988) undergo the transition during 8–16-cell stage. These differences in the onset of the MZT may reflect an inter-play between transcription factors, the transcriptional machinery, and chromatin structure as well as DNA accessibility (Temeles et al., 1994; Thompson et al., 1998). Together, these variables regulate the mechanisms responsible for the transition to embryonic control.

More recent studies have observed a minor activation of the embryonic genome in cattle prior to the major activation at the MZT (Viuff et al., 1996; Memili and First, 1999). The observations by Viuff et al. (1996) included RNA synthesis in the 2- and 4-cell stage embryo that was characterized by a lack of a well-defined transcriptional peak during the second cell cycle. Memili and First (1999) were able to detect embryonic genome activation earlier yet, i.e., in the 1-cell zygote. Both of these studies pointed out the necessity for minor genome activation early on to achieve later developmental success.

Another recent study (Hyttel et al., 2000) explored embryonic genome activation for rRNA genes in cattle and pig embryos. The focus of the research was to correlate aberrations in the genome activation machinery, the nucleoli, with cell cycle arrest at later developmental stages. The results showed that the nucleoli became structurally recognizable with rRNA gene activation occurring in blastomeres of in vivo-derived embryos at the end of the third- and fourth-cell cycles for pigs and cattle, respectively. Interestingly, in vitro-derived cattle and pig embryos displayed abnormal nucleolus formation, which was associated with deficient rRNA gene activation. In turn, these problems with rRNA gene activation result in developmental abnormalities later, which may include cleavage arrest or certain deleterious phenotypes commonly associated with offspring derived from in vitro produced embryos.

The ordered series of controls and checkpoints that comprise the cell cycle are the driving force behind cell proliferation. Of the checkpoints within the cell cycle, the transition from G2 to M is controlled by a conserved group of participants. The key participant is maturation promotion factor (MPF; Labbe et al., 1989). One subunit of MPF is a 34 kDa protein termed cdc2, which functions as the catalytic domain of the dimer (Arion et al., 1988). The other subunit, cyclin B1, is the regulatory subunit of the dimer (Pines and Hunter, 1989). Regulators of MPF include wee1 kinase (Gould and Nurse, 1989) and cdc25 phosphatase (Dunphy and Kumagai, 1991), which is also known as STRING in *Drosophila* (Nurse, 1990). Wee1 kinase activity favors a G2, highly phosphorylated, inactive form of MPF, while cdc25 activity favors a hypophosphorylated, highly active, M-phase form of MPF (Murray and Kirschner, 1989). Even though three isoforms of cdc25 (a, b, and c) have been identified in mammals (Pines, 1995), the focus of this study will be on the G2/M regulator cdc25c.

In an effort to characterize some of the potential molecular mechanisms involved in the reduced development associated with in vitro matured–in vitro fertilized (IVM–IVF) derived embryos (Prather and Day, 1998), our group investigated the quantities of mRNA for one of the positive regulators of mitotic induction, cyclin B1, during the porcine MZT (Anderson et al., 1999). A gradual decline in cyclin B1 levels in all embryos during the 4-cell stage was observed, with no evidence for de novo synthesis of message. In addition, no effect of exposure to α-amanitin was found for the quantity of cyclin B1 message during the 4-cell stage. Thus, maternal cyclin B1 mRNA degradation occurs during the 4-cell stage, with no detectable embryonic-derived transcripts present.

The dependency of the *Drosophila* MZT on maternal STRING degradation, the problems associated with porcine embryonic development, which include substantial in vivo- (Pope and First, 1985) and in vitro-derived (Prather and Day, 1998) embryonic loss, and our previous cyclin B1 study prompted us to shift our focus to cdc25c, an essential positive regulator of mitotic entry. This, our second study on transcriptional control over the lengthy 4-cell stage pig embryo was undertaken to determine the role that both maternal and embryonic derived cdc25c transcripts play in the timing of the porcine MZT. A comparison of cdc25c transcript levels in both in vivo- and in vitro-derived 4-cell embryos, as well as in other cleavage stages, was made to determine the timing and magnitude of the shift to embryonic transcript production. Differences in cdc25c transcript quantity as well as the interactions between maternal message degradation and initial genome activity may aid in our understanding of the large discrepancy in developmental competence observed for the two embryo types, in vivo- and in vitro-derived.

MATERIALS AND METHODS

General

All chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. The techniques used to investigate porcine cdc25c mRNA levels are based on those described previously (Anderson et al., 1999).

Animals

Experiments involving the use of animals were conducted in accordance with protocols approved by the University of Missouri Animal Care and Use Committee. The checking of gilts for estrus, breeding, and recovery of 2-cell embryos were previously described (Machaty et al., 1998; Anderson et al., 1999). All recovered embryos were pooled for later random distribution to replicates. The oviductal flush and embryo culture medium was Whitten's Medium (WM) plus 0.3% BSA (Schoenbeck et al., 1992).

Embryo Culture and Timing

In vivo-derived 2-cell stage embryos were cultured in vitro according to standard protocols (Schoenbeck et al., 1992). To monitor when individual 2-cell embryos cleaved, embryos were briefly checked every two hours, with time of cleavage to the 4-cell stage noted. When cleavage to the 4-cell stage occurred, embryos were individually moved to a different drop of medium and randomly assigned to one of three treatment groups: Group 1 were control embryos cultured in WM for 5-, 10-, 18-, 25-, or 33-hr P4CC. Group 2 embryos were cultured in WM plus the RNA polymerase inhibitor, α-amanitin (20 μg/ml), over the same time periods as the control embryos. Group 3 were additional embryos cultured in WM + 20 μg/ml α-amanitin from either: 1) 5–33-hr P4CC; 2) 10–33-hr P4CC; 3) 18–33-hr P4CC; or 4) 25–33-hr P4CC. Each replicate consisted of 14 individual 4-cell embryos from the three treatment groups plus RT-cPCR controls.

In Vitro-Derived Embryos

Porcine ovaries were collected at a local abattoir (Excel Co., Marshall, MO), transported to the laboratory, and oocytes were aspirated (Anderson et al., 1999). IVM-IVF (Abeydeera and Day, 1997), WM culture, assignment of individual embryos to one of the three treatment groups, and embryo storage was as described before (Anderson et al., 1999).

Other In Vivo-Derived Embryos and Oocytes

In addition to the 4-cell stage embryos used in this study, three other periods of early in vivo embryonic development were also investigated. These different stages were cultured in vitro for less than 2 hr, and represent reference points outside the MZT. The three included metaphase II (MII) oocytes (n = 14), 2-cell (n = 12), and late compact morulae (LCM; n = 14) stage embryos.

Embryo RT

Following the isolation of poly (A) RNA from individual embryos/oocytes (Anderson et al., 1999), the RT reactions (20 μl) were performed with components prepared in large mixtures beforehand to help minimize variation.

For each replicate of 14 in vivo- or in vitro-derived embryos, three RT-PCR controls were included when the embryos were collected, timed, and stored prior to mRNA isolation. The three controls that were subjected to the same procedures as the embryos were: 1) a positive RT-control that consisted of day 17 porcine embryo RNA; 2) a DNA contamination control identical to the positive RT-control except for omission of reverse transcriptase from its mix; and 3) a negative control that contained only diethylpyrocarbonate-treated PBS (DEPC-PBS).

Primers

The following primers were synthesized (Gibco-BRL, Gaithersburg, MD), and used in all experiments. Sequences are read 5′ to 3′. Primers for 403-bp cdc25c: 1) cdc25c-upper (U), TCG AAG CCA GAA CAA AGC AT; 2) cdc25-lower (L), AAA CCC AGT GAG AGA AAA AT. Primers for 343-bp cdc25c: 1) Nested (N)-cdc25c-U, CAG ATT GCC TTA CTC GTG AA; 2) N-cdc25c-L, GAT GGC CTC TGA AAA GAC TT. Cdc25c-"40-mer", GAT GGC CTC TGA AAA GAC TTG AGC CAG CTT GAG GAT TTC.

Clones

By using total RNA derived from whole day 17 porcine embryos, a 403-bp cdc25c clone was amplified by RT-PCR. The primers for this sequence were derived from a previously published porcine cdc25c report (GenBank accession no. X78317; Cui et al., 1995), and are described above. The 403-bp cdc25c product was gel purified, subcloned, and identity confirmed using the techniques described by Anderson et al. (1999).

Our 403-bp cdc25c clone exhibited 100% identity with the X78317 complete porcine cdc25c sequence, and exhibited 74% identity with a human cdc25Hs mRNA (GenBank accession no. M34065; Sadhu et al., 1990). The N-cdc25c primer sequences and associated 343-bp amplified cDNA product, the 256-bp DNA competitor, and the digoxigenin (DIG)-labeled Southern hybridization probe are derived from the sequence of the original 403-bp clone.

Competitor Production and Use

The 256-bp truncated cdc25c competitor was generated by using the cdc25c "40-mer" and N-cdc25c-U primer in PCR on the original cdc25c subclone. The 256-bp competitor was then diluted from a stock of known concentration, and the working stock concentration of 200-attograms (ag)/μl was used in each individual cPCR.

Standard-Curve Dilution Series

By using the subcloned 343-bp cdc25c DNA product as starting material, a log-dilution series with a concentration range (0.023–6.90-fg/μl) that corresponded to the amplification profile observed for individual embryos during cPCR was produced. The use of these DNAs along with 200 ag of the truncated 256-bp competitor formed the standard curve (Fig. 1).

Embryo and Standard-Curve cPCR

The 343-bp embryo cDNA was co-amplified along with 200 ag of the truncated, 256-bp, cdc25c competitor using the N-cdc25c-U/L primer set in individual reactions (Anderson et al., 1999). By using the three different volumes of embryo cDNA in cPCR procedures, as outlined below, allowed for an amplification profile to be determined for each individual embryo at its respective time point (Anderson et al., 1999).

Standard-Curve Generation and Use

The techniques used to obtain the results from each replication (n = 8, in vivo; n = 7, in vitro) are described by Anderson et al. (1999). Averaging the 343:256-bp

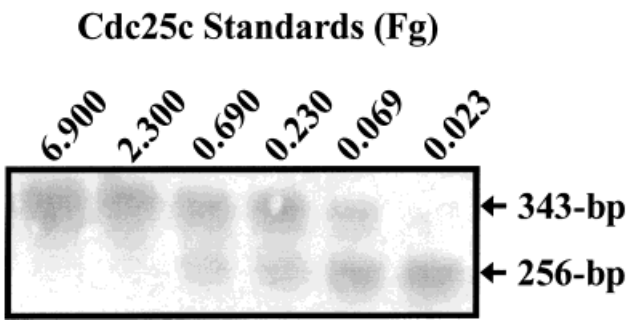

Fig. 1. Southern hybridization of cdc25c standard-curve: competitor dilution series. The 343-bp cdc25c standards (0.023–6.9 fg/μl) were co-amplified over the exponential portion of the curve with 200 ag/μl of the 256-bp truncated cdc25c mimic using cPCR.

cdc25c ratio for each specific cdc25c standard across all replicates generated standard-curve values. After log-transformation of each standard concentration, the average ratio along with the log-transformed concentration of each standard was used to generate a line-of-best-fit and its regression equation of $Y = 2.1857 + 1.4007X$, with $R^2 = 0.952$. This equation was used to calculate the quantity of cdc25c mRNA present at the specific time points.

Ratios were generated for the 3-cDNA dilutions (5-, 2.5-, and 1.25-μl of original RT product) of each specific embryo time point. These adjusted embryo mRNA values had to adhere to the following criteria before being accepted as valid points: 1) Fall within the middle 80% of the standard-curve values. 2) Parallelism: a compensated value (i.e., multiplied by appropriate dilution factors) should correspond to at least one of the other two cDNA-dilution values for a given time point within a replicate. If parallelism was observed, then the average of the parallel concentrations was taken and used in the analysis (Anderson et al., 1999). 3) Nonparallel cDNA concentrations: in the situation where no parallelism was observed for a specific time point within a replicate, the average concentration of those points which displayed decreasing ratios with decreasing original cDNA volume (i.e., volume of cDNA originally added to cPCR mix) were used in the cdc25c mRNA calculations. 4) Abnormal competition: if embryo ratios increased or remained unchanged with decreasing cDNA volume for a specific time point, these ratios were not used in the calculation of cdc25c mRNA quantity. Of the 15 replicates with 14 embryos each (210 total embryos), 22 embryos failed to meet the above criteria and were not used in the calculations. All calculations involving the embryo cDNA ratios and corresponding mRNA concentrations were adjusted to be the equivalent of one embryo.

Statistical Analysis

The General Linear Model of the Statistical Analysis System (SAS; SAS Institue, Cary, NC) was used to evaluate treatment effects. The effects of differing periods of exposure to α-amanitin and embryo source (in vitro vs. in vivo) on cdc25c mRNA levels were evaluated by split-plot analysis of variance. The effect of embryo source was tested using replicate (data from individual replicates) within embryo source as the whole plot error term. The subplot analysis used the residual error term to evaluate the effect of differing periods of exposure and related interactions. The effect of continuous exposure to α-amanitin on cdc25c gene expression over time also was evaluated by split-plot ANOVA. The effects of α-amanitin and embryo source were tested using replicate within embryo source and α-amanitin treatment as the error term. Changes in mRNA levels over time and related interactions were evaluated in the subplot analysis, using the residual error. Least significant difference intervals were used for multiple comparisons of means.

RESULTS

Cdc25c mRNA Levels in Porcine Embryos

The comparison of embryo type (in vivo vs. in vitro) was not significant ($P > 0.05$). The control embryos had a higher level of cdc25c mRNA at 5-hr P4CC (46.1-zeptomoles (zmol)) when compared to the 10-, 18-, 25-, and 33-hr P4CC groups (39.7-, 38.1-, 40.1, and 32.7-zmol, respectively; $P < 0.05$; Fig. 2). The embryos treated with α-amanitin from time of 4-cell-cleavage to either 5-, 10-, 18-, 25-, or 33-hr P4CC contained 51.2-, 30.2-, 38.5-, 28.7-, and 31.9-zmol cdc25c mRNA per embryo, respectively (Fig. 2). For group 1 and 2 embryos at each specific time point, α-amanitin had no effect on mRNA levels ($P > 0.05$).

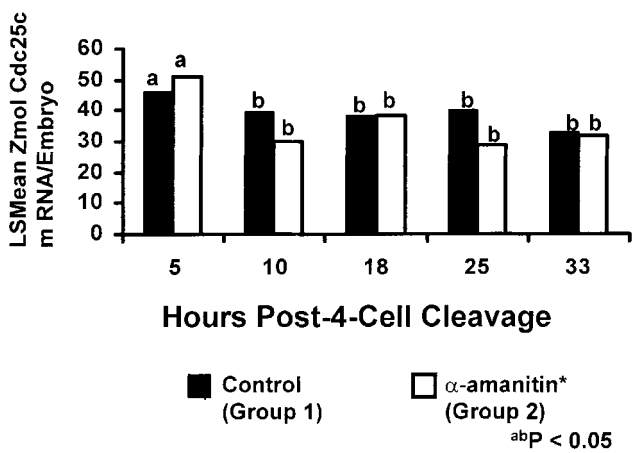

Fig. 2. LSMean zeptomoles cdc25c mRNA in 4-cell stage control (group 1) and α-amanitin (group 2) embryos. In vivo- (8 replications) and in vitro- (7 replications) derived embryo LSMeans were pooled together due to lack of embryo-type effect ($P > 0.05$). All LSMeans were log transformed to correct for heterogeneity of variance. *LSMeans for the combined treatments within a time point were higher for the 5 hr group as compared to the other time points ($P < 0.05$); the LSErrors for these comparisons were 4.05, 4.28, 4.22, 3.91, and 4.09 for the 5 through 33 hr time points, respectively.

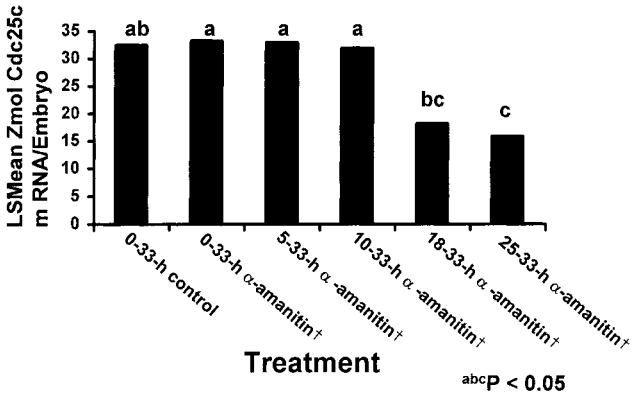

Fig. 3. LSMean zeptomoles cdc25c mRNA in single embryos (group 3) cultured for various lengths of time ending at 33-hr P4CC in both control and α-amanitin containing medium. In vivo- (8 replications) and in vitro- (7 replications) derived embryo LSMeans were pooled together due to the lack of embryo-type effect ($P > 0.05$). All LSMeans were log transformed to correct for heterogeneity of variance. LSMeans bearing different lowercase letters can be compared, and differ significantly ($P < 0.05$). The (†) designation indicates that α-amanitin exposure began at either 0-, 5-, 10-, 18-, or 25-hr P4CC and continued until 33-h; the LSErrors for these comparisons were 4.70 for the control and 4.69, 4.83, 2.53, 4.30, and 4.48 for the 0–33, 5–33, 10–33, 18–33, and 25–33 hr treatments, respectively.

The embryos treated with α-amanitin for various windows of time (group 3 embryos), including 0–33-, 5–33-, 10–33-, 18–33-, or 25–33-hr P4CC, were found to contain 31.9-, 32.0-, 31.6-, 18.8-, and 16.6-zmol cdc25c mRNA per embryo, respectively (Fig. 3). A decline in the quantity of cdc25c mRNA was observed in those embryos treated with the inhibitor from either 18–33- or 25–33-hr P4CC ($P < 0.05$) as compared to the other three time point windows. When the α-amanitin treated embryos were compared to the 0–33-hr control treatment (Fig. 3), the quantity of cdc25c mRNA in the 25–33-hr P4CC α-amanitin-treated embryos was reduced ($P < 0.05$).

In Vivo-Derived MII Oocyte, 2-Cell Stage, and LCM Embryo Reference cPCR

Following cPCR of in vivo-derived MII oocytes (n = 14), 2-cell (n = 12), and LCM (n = 14) embryos, the ratios obtained from comparing the oocyte- or embryonic-derived 343-bp cdc25c to the 256-bp introduced cdc25c DNA competitor fell in the range of the standard curve. The LSMean value of mRNA in the MII oocytes was 57.1 ± 12.6-zmol per embryo from the 11 individuals that met the hierarchy of selection criteria outlined above. The LSMean quantity of mRNA present in the early-2-cell embryos was 44.0 ± 12.1-zmol per embryo from all 12 individuals originally collected as all met the selection criteria. Of the 14 LCM originally collected and used in cPCR, 12 filled the hierarchy of selection criteria with an observed LSMean of 87.6 ± 12.1-zmol per embryo. There was no difference in the quantity of cdc25c mRNA observed in the MII oocytes and 2-cell stage embryos ($P > 0.05$). However, the quantity of cdc25c transcript present in the LCM was higher than the levels observed in the 2-cell embryos ($P < 0.01$), with a tendency to be higher as compared to the MII oocytes ($P = 0.07$).

DISCUSSION

We have described the changes in cdc25c mRNA quantity in both in vivo- and in vitro-derived 4-cell stage porcine embryos. Our quantitative RT-cPCR technology was used on individual embryos to compare zeptomolar (10^{-21}-moles: 10,000–53,000 molecules per embryo) amounts of cdc25c transcript at different time points during the 4-cell stage and at other developmental stages before and after the time of the MZT. The major findings of this work were that both in vivo- and in vitro-derived 4-cell embryos contain similar quantities of cdc25c transcript with a shift to embryonic message production occurring between 10- and 18-hr P4CC. In addition, this shift to embryo derived cdc25c message was accompanied by degradation of maternal transcripts that appeared to be controlled by embryonic genome activity.

In the culture system used, both embryo types had the ability to progress through the 4-cell block stage, and continue development. However, in vivo-derived embryos consistently displayed higher cleavage, development, and blastocyst rates during culture as compared to those that were in vitro-derived (data not shown). Since both in vivo- and in vitro-derived 4-cell embryos had similar levels of cdc25c transcript, it would appear that message levels of this cell cycle controller are not responsible for the poorer development associated with laboratory produced embryos. Therefore, deficiencies in other sources of regulation that may be tied to culture conditions could be responsible for the poor development associated with IVM-IVF embryos. Potential sources of such regulation that have been shown to be active in the changes associated with MZT initiation include: 1) Translational initiation (Bachvarova, 1992); 2) translational activation of pre-existing maternal transcripts (Bolton et al., 1984); 3) post-translational modifications (Van Blerkom, 1981); 4) polypeptide turnover (Howlett, 1986); and 5) translation of newly synthesized, embryonic-derived transcripts (Flach et al., 1982).

The second finding in this current study was the presence of zygotic derived cdc25c transcripts after 10-hr P4CC along with an apparent α-amanitin effect on maternal cdc25c degradation. The presence of embryonically derived transcripts are shown in Figure 3 by a decrease in the quantity of cdc25c present in the 25–33-hr P4CC α-amanitin group when compared to 0–33-hr control embryos and the 5–33- and 10–33-hr P4CC inhibitor treatments. The data obtained from the α-amanitin treated embryos over the window from 18–33-hr P4CC showed only a tendency ($P = 0.09$) for a decrease in cdc25c transcript quantities when compared to the control embryos.

The apparent effects of α-amanitin repression on maternal RNA degradation are also shown in Figure 3

by comparison of the 0-33-hr α-amanitin treated embryos to the other groups of α-amanitin treated embryos. The maintained levels of cdc25c transcript in the 5- and 10-hr groups as compared to the 0-33-hr α-amanitin group are in sharp contrast to the reduced levels of maternally derived cdc25c transcript in the 18- and 25-hr groups. Culture in α-amanitin from 0-33-hr P4CC did not allow for activation of the zygotic genome, but resulted in significantly higher levels of message at 33-hr P4CC when compared to the 18- and 25-hr groups, implying that zygotically derived transcripts were required for degradation of maternal cdc25c. This dependence on embryonic genome activation for maternal cdc25c (STRING) degradation was also observed by Edgar and Datar (1996). The findings of this study were that accumulation of newly synthesized embryonic gene products controlled STRING levels at the MZT and beyond.

The decrease in cdc25c mRNA observed before the MZT (10 hr group; Fig. 2) in the present study was most likely the result of maternal degradation driven by minor activation of the embryonic genome over the 2 hr window of variability present during the timing of cleavage from 2- to 4-cell stage. This 2 hr interval represents a time that corresponds to a minor genome activation period described by Schoenbeck et al. (1992). This initial minor activation coupled with the unknown rate or kinetics of α-amanitin action to block RNA polymerase activity provides the best explanation for the observed decline in cdc25c mRNA levels early on during the 4-cell stage.

The quantities of cdc25c transcript determined in this study for MII oocytes, 2-cell embryos, and LCM fell within the range of the cdc25c standard-curve, and as a result, values could be determined. In our previous study (Anderson et al., 1999), cyclin B1 mRNA values could not be calculated for these same time points due to the very high levels of cyclin B1 transcript present, which resulted in the ratios falling outside the standard curve range. The relative levels of cdc25c transcript in the present study were ~1,000-fold less (10^{-21}moles vs. 10^{-18} moles) than cyclin B1 levels.

In the present study, the quantities of cdc25c transcript in MII oocytes and 2-cell stage embryos were the same, with only the LCM exhibiting increased transcript levels over the 2-cell stage embryos. A tendency for an increase in LCM cdc25c transcript levels was observed when compared with MII oocytes. The LCM most likely display increased cdc25c message levels due to heightened transcription of the embryonic genome. The similar levels of cdc25c message in MII oocytes and the 2-cell stage embryo, which are comparable to the levels observed over the 4-cell stage, can be explained by maternal transcript levels being set in the oocyte prior to ovulation with changes resulting only as a consequence of degradation or translation (Kidder, 1992; Henrion et al., 1997). Also, cdc25c protein levels (Millar et al., 1991; Girard et al., 1992) do not cycle or fluctuate like cyclin B1 (Arellano and Moreno, 1997), so large variation in cdc25c transcript levels are not necessary. In addition, the existence of a wide range of cyclin B1 protein and associated activity levels over the course of the cell cycle is most likely controlled by a multitude of post-transcriptional modifications (Mitra and Schultz, 1996), and may also help explain why the quantities of cyclin B1 transcript are at least 1,000-fold higher than cdc25c message at the same time points.

The results obtained in previous studies (Jarrell et al., 1991; Schoenbeck et al., 1992) that found maternal control of porcine development up to 16-hr P4CC are supported by results from the present study. Jarrell et al. (1991) found a decreasing rate of uptake and incorporation of radiolabeled-methionine from the unfertilized oocyte to the early 4-cell stage. This finding was proposed to represent the relationship between the increasing levels of maternal mRNA degradation over this time, and the decreased number of transcripts available for translation. This apparent relationship prompted the investigation of the 4-cell stage pig embryo in greater depth (Schoenbeck et al., 1992), which described an α-amanitin sensitive qualitative shift in the proteins expressed from ~12- to 16-hr P4CC. This information along with cleavage data obtained by culturing embryos for various lengths of time in α-amanitin, suggested that by 24-hr P4CC, at the latest, the porcine embryos had the transcripts required for successful cleavage to the 8-cell stage.

The final topic that needs to be addressed is the limitations of the quantitative RT-cPCR technique described above as compared with more recently developed real-time RT-PCR systems. The current study as well as our previously described cyclin B1 work (Anderson et al., 1999) was initiated either prior to the existence of or during the infancy of the real-time technology in use today. The quantitative RT-cPCR technique used in this study does have more limitations than real-time PCR including decreased sensitivity and increased variability, which are both due to the RT-PCR itself (Freeman et al., 1999; Bustin, 2000). In addition, the multiple steps required for the visualization, assessment, and documentation of the information obtained from the embryos used in this study following cPCR completion are eliminated with the currently available real-time techniques that incorporate fluorescent probes into the amplification reaction. The elimination of these post-PCR steps is not only valuable in terms of the time saved, but also as an improvement to quantitation through the removal of errors commonly associated with the techniques (Freeman et al., 1999). Together, the combined variation for the steps associated with quantitative RT-cPCR can be as high as 14%, while real-time PCR strategies have measured variation that typically range from 0 to 5% depending on the system used (Bustin, 2000). However, we believe that the steps described in this study taken to minimize the variation between replications and maintain a reasonable level of reproducibility, even though embryo variability could not be controlled, were the most reliable techniques available at the initiation of these experiments.

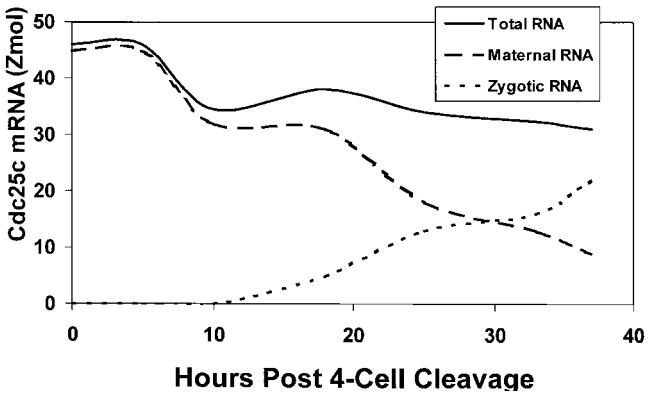

Fig. 4. Proposed timing of porcine cdc25c ZGA during the 4-cell stage. Cdc25c total mRNA (solid line), maternal mRNA (long dashed line), and zygotic-derived RNA (short dashed line) are depicted. This graph is based on Figures 2 and 3 depicting the proposed timing for the degradation of maternal message as well as the onset of RNA synthesis for cdc25c.

CONCLUSIONS

The conclusions to be drawn from this study are that: 1) Cdc25c message levels over the 4-cell stage follow a similar pattern in both in vivo- and in vitro-derived pig embryos; and 2) Cdc25c message quantities appear to be regulated by a mechanism that includes initial activation of embryonic transcription from 10- to 18-hr P4CC and maternal message degradation controlled by genome activity over the same period (Fig. 4).

Even though we did not observe a role for cdc25c transcripts, or lack thereof, at the MZT in the more poorly developing in vitro-derived embryos, the finding that maternal messages were regulated by embryo derived transcripts, and most likely their post-transcriptionally modified proteins, highlights the magnitude of the complexity associated with genome activation in the pig.

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